FINAL REPORT

Rapidly Degradable Pyrotechnic System

SERDP Project WP-1622

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List of Acronyms

ATCC:	American Type Culture Collection
Amp:	Ampicillin
BamHI:	Restriction Endonuclease
BTA1:	Hydrolase Encoding Gene
CYTEXP1:	E. coli expression vector
epPCR:	error prone PCR
IED:	Improvised Explosive Device
IST:	Infoscitex Corporation
Kan:	Kanamycin
LCP:	Liquid Crystalline Polymer
LB:	Luria Broth
LB-Amp:	Ampicillin-Luria Broth
NdeI:	Restriction Endonuclease
OmpA:	Outer Membrane Protein Gene
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PET:	Polyethylene-terephthalate
PLA:	Poly-Lactic Acid
pNPP:	p-Nitrophenyl Palmitate
RDPS:	Rapidly Degradable Polymer System
SERDP:	Strategic Environmental Research and Development Program
TAMU:	Texas A&M University
TfH:	Thermophilic hydrolase from *Thermobifida fusca*
TLCP:	Thermotropic LCP
Δ:	Deletion of Gene

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1. EXECUTIVE SUMMARY

In this SERDP proof-of-concept project, Infoscitex established feasibility of using enzymatic approaches to develop a rapidly degradable, environmentally benign, pyrotechnic system

Training grounds and battlefields are being polluted by the remains/debris of detonated/fired or, in some cases, unexploded pyrotechnic devices. The most abundant source of pollution comes from the outer casing-housing that is usually constructed from aluminum or steel protected by a corrosion resistant coating containing hexavalent chromium and cadmium. Adverse environmental and health affects are associated with these coatings. Decommissioning training fields contaminated with pyrotechnic debris requires significant cleanup and remediation

The focus of this SERDP effort was to develop biocatalyst for hydrolysis of certain liquid crystalline polymers (LCP); thus these polymers could be used as a replacement for metal casings. The program encompassed three main activities:

- Generation of a microbial strain that would be able to produce LCP degrading enzymes
- Trial LCP hydrolysis with the LCP degrading enzymes
- Estimating the degree of LCP degradation

The outcomes of the Phase I activities included the following:

- Several *E. coli* clones capable of elevated production of hydrolases active on the LCP-like structures were generated using genetic engineering
- The *E. coli* clone culture supernatants were evaluated for LCP degradation. No TLCP mass loss was observed with these clones
- Some deterioration of the mechanical properties of the selected LCP was observed at 50 °C and using supernatants of *Thermobifida* cultures.
- No deterioration of the TLCP mechanical properties was observed during the experiments performed at ambient temperatures using supernatants of *Thermobifida* cultures.

2. OBJECTIVE

The overall objective of the proposed effort was to develop an approach to environmentally benign and economical pyrotechnics material system (structural polymer and degradation agent) for producing a high strength, non-corroding, highly inert, environmentally safe, extended/indefinite shelf life pyrotechnic housing-casing that can also be readily degraded on-demand using specially tailored and safe enzymes. The specific goals for demonstrating the feasibility/viability of this innovative proposed material system included:

- Demonstrating the selected structural polymer has sufficient mechanical properties to replace the presently used metallic housing/casing materials with no negative impact on pyrotechnic performance, shelf life, weight and life cycle cost.
- Develop a specially tailored enzyme and demonstrate its ability to degrade the structural polymer.

Overall, the Infoscitex team planned to achieve these goals by:

- Developing enzymes for LCP degradation
- Optimizing the degradation process conditions and controls
- Integrating these enzymes in the body of the pyrotechnic housing-casing

3. BACKGROUND

Infoscitex explored the feasibility of enzymatic degradation of polyethyleme-terephthalate (PET) based thermotropic liquid crystal polymer (TLCP), which is corrosion resistant and offers excellent mechanical properties to serve as a potential drop in replacement for the presently used metallic based pyrotechnic housings/casing. The second material and primary focus of the program effort was the improvement of a newly emerging enzyme, a thermophilic hydrolase formed from *Thermobifida fusca* (TfH). Hydrolase genes were mutated to develop clones expressing the highest PET hydrolase activity and ability to impair the TLCP mechanical properties of and degrade it into inert and environmentally safe by-products.

3.1. Environmental Relevance

Battlefields and training fields are being polluted by the remains/debris of activated pyrotechnic devices or in some cases unexploded devices. It is estimated that at a minimum 500,000 to

1,000,000 devices are used each year for training and in actual battlefield encounters. Military pyrotechnics cover a wide range of devices. The following list describes some of the devices that can create battlefield debris:

- Photoflash Cartridges – both practice and live 4 second and 6 second delay devices
- Flares – for aircraft, ballistic aerial target and surface applications. These devices can be equipped with or without parachutes and be used for countermeasures, infrared tracking, alert mechanisms for enemy infiltration via trip wires, illumination of battlefields and aircraft runways, target marking and aerial reconnaissance.
- Signals – these devices can be equipped with or without parachutes, display a wide range of colors (red, green, white and yellow in both a single and double star pattern) and are used for aircraft illumination, ground illumination, distress alert in both foliated and non foliated environments, and for the generation of smoke
- Simulators – for atomic explosion, artillery flash, hand grenades, launching of anti-tank, guided missiles and rockets, air burst projectiles, ground burst projectiles, tank and main gun signature, and direct and indirect fire cue.
- Miscellaneous devices covering a range of specialty applications

A fairly significant and common source of pollution associated with these devices comes from the outer casing-housing that is usually constructed from aluminum or steel protected by a corrosion resistant coating containing chromium and cadmium. These items persist in the environment and their protective coatings can release heavy metals into the soil via leaching that subsequently migrate into the water table. Clean up costs for eliminating/removing these pollutants from training fields represents a significant burden for DoD installations. Some military training fields/firing ranges have been or will be closed due to pollution related issues. Such closures will clearly have a negative impact on our ability to properly train our military personnel and thus could reduce force readiness. In addition, used pyrotechnic housings/casings left on a battlefield can become a tactical hazard. For example, in Operation Iraqi Freedom, used casings have been converted into improvised explosive devices (IEDs) by insurgents that in turn were and are being used against U.S. Forces. Clearly a new pyrotechnic housing/casing system that will quickly degrade and render the casing into an unusable container, and with further time fully degrade into inert, unrecognizable and environmentally safe materials will solve a variety of problems facing the DoD. The IST Phase I addressed the DoD need for a environmentally compliant casing system through the development of the Rapidly Degradable Polymer System (RDPS).

3.2 Approach

3.2.1 Overview of IST's Solution Technical Approach

IST's RDPS solution (Figure 1) utilizes an inert (non-corrosive) polymer, which is also one of the strongest commercially-manufactured polymers, as a replacement for the chromium and cadmium coated aluminum and/or steel housings. We will combine this material with a unique prepackaged desiccated enzyme that will be released and activated immediately after the pyrotechnic device functions. With time the enzyme will degrade the polymer, making the housing/container relatively unusable in the short term and in the longer term reducing it into inert and environmentally safe by-products. If needed, a secondary enzyme activating material such as water combined with environmentally safe freezing point depressant salts can be added to and kept separate from the desiccated enzyme using encapsulation technology or segregated packaging. When the pyrotechnic device is fired the protective enzyme/additive packing fails, releasing the materials where the mix and begin to degrade the pyrotechnic housing/casing.

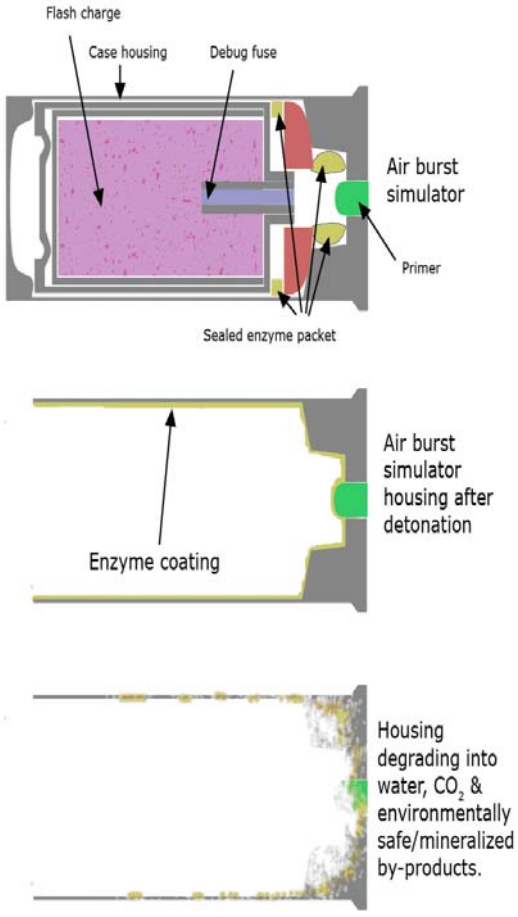

Figure 1 – IST's RDPS

3.2.2 Rationale for Selecting Thermotropic Liquid Crystal Polymers (TLCP) over Naturally Biodegradable Polymers

Rather than use naturally biodegradable polymers for constructing the pyrotechnic housing/container, IST's approach utilized the use of an exceptionally high strength and highly inert TLCP that will only degrade on demand by the action of an enzyme that is released and activated immediately after the pyrotechnic device is activated/used. Our innovative approach for mitigating/eliminating the negative environmental impact of spent pyrotechnic assemblies is illustrated in Figure 1. We have selected TLCPs over biodegradable polymers due to the inherent deficiencies that biodegradable polymers have, especially in this application. These deficiencies include:

- Inherent biodegradability hinders shelf life. Balancing the contradicting requirements for degradability and long shelf life is problematic when using naturally biodegradable polymers/materials. A biodegradable housing will require protective packaging to provide an extended shelf life. This adds to logistic burdens, cost, increased difficulty in use and increased risk the device will malfunction if the protective packaging becomes compromised.
- Very low mechanical properties. Biodegradable polymers have tensile strengths that are 1-2 orders of magnitude lower than aluminum or steel (Table 1), the standard materials used to fabricate pyrotechnic outer housings. They can not compete structurally with traditionally used materials unless they are combined with structurally reinforcing continuous fibers. This type of construction significantly increases the manufacturing cost by 3 to 10 fold and compromises the ability to achieve complete degradability.

3.2.3 Thermotropic Liquid Crystal Polymer (TLCP)

TLCPs as listed in Table 1 are a unique class of wholly aromatic polyester polymers that provide exceptional mechanical properties, equaling or exceeding the properties of presently used pyrotechnic housing materials such as aluminum and steel. TLCPs are unique because they exhibit a partially ordered melt state that is intermediate between a three-dimensional ordered crystalline state and the disordered or isotropic fluid state. As a consequence of the molecular ordering, TLCPs are anisotropic (i.e., their properties are a function of molecular direction). IST engineers are experts in processing TLCPs and have developed processing equipment that transforms the localized short range ordered domains of the TLCP melt into a long range ordered structure that provides the excellent mechanical properties needed for this application. Structurally, most commercial TLCPs as shown in Figure 2 consist of rigid mesogenic monomer units connected with either flexible spacers or "kink structures" to make them tractable and

processible. The high degree of molecular order that can be achieved with the TLCP molecules allows this material to attain a very tight packing density similar to a log jam in a river. TLCPs derive their outstanding properties from this tightly packed rigid-rod formation which at a supra-molecular level results in a structure that is self-reinforced through the strong interaction of electron deficient and electron rich carbon rings. Because of this self- reinforcement, TLCPs do not suffer from microcracking. When this tight packing property is combined with the TLCP low solubility, an ideal structure is formed for providing excellent barrier properties. In addition, because the diameter of the TLCP microfibrils is less than 1.0 µm, the TLCP is very homogenous, which is critical for retaining small molded features.

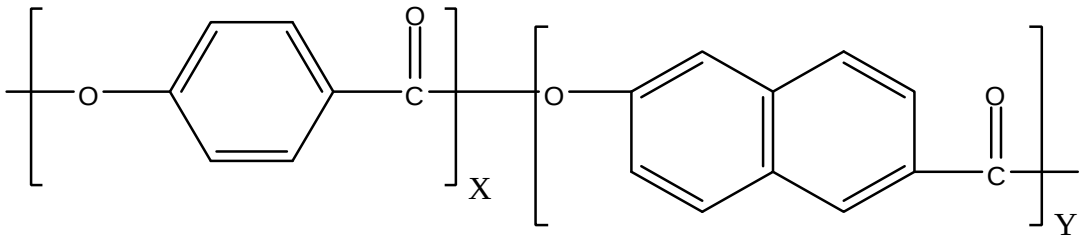

Figure 2 – Chemical Structure of a TLCP

Table 1 – Comparison of a Typical TLCP's Properties with Various Engineering Materials

Material	Density (g/cm^3)	Tensile Strength (KSI)	Specific Tensile Strength ($KSI/g/cm^3$)	Tensile Modulus (MSI)	Specific Tensile Modulus ($MSI/g/cm^3$)
Vectran TLCP	**1.4**	**412**	**294**	**9.4**	**6.7**
Steel	**7.8**	**145**	**19**	**29**	**3.5**
Titanium	4.5	134	30	16	3.5
Aluminum	**2.8**	**67**	**24**	**10**	**3.5**
Glass Fiber	2.5	246	98	10	4.0
Carbon Fiber	1.9	228	120	55	29.0
PLA Biodegradable Polymer	**1.2**	**8.7**	**7.25**	**0.5**	**0.42**
PLA after 8 weeks weathering	**1.2**	**4.9**	**4.1**	**0.39**	**0.33**

(Note: LCPs have superior tensile strength, specific tensile strength and specific tensile modulus than traditionally used pyrotechnic outer housing materials – steel and aluminum. Compared to a traditional biodegradable polymer such as PLA, TLCPs have a tensile strength that is 47 times higher and a tensile modulus that is 18.8 times higher. Biodegradable polymers such as PLA also lose significant mechanical properties when exposed to natural weathering. After 8 weeks of weathering, the tensile strength and tensile modulus of PLA will drop 43% and 16%, respectively.)

In summary TLCPs have outstanding mechanical properties at high temperatures, excellent chemical resistance, inherent flame retardancy and good weatherability. TLCPs are exceptionally inert. They resist corrosion (protective corrosion resistant coatings will not be required, thus eliminating the need for heavy metals such as Cadmium and Chromium), and they also resist stress cracking in the presence of most chemicals at elevated temperatures, including aromatic or halogenated hydrocarbons, strong acids, bases, ketones, and other aggressive industrial substances. Hydrolytic stability in boiling water is also excellent. The major advantages of TLCPs include: lightweight, high heat resistance, flame retardant, chemical resistance, dimensional stability, near hermetic barrier properties, low CTE, heat aging resistance, low processing viscosity, and weldability. Because of their excellent mechanical and corrosion resistant properties, LCPs have the best potential for replacing the presently used non-corrosion resistant metal based pyrotechnic casings without the need for any protective coatings. More specifically, the Vectran TLCP (Table 1) presents an excellent choice and was selected for design on the new RDPS.

3.2.4 Enzymatic Depolymerization

Synthetic polymers such as our proposed TLCP have become common substitutes for natural materials, providing greater shelf stability and durability. Many of these plastics, particularly those with excellent mechanical properties are inheritably resistant to chemicals and because naturally occurring organisms capable of degrading synthetic polymers have not evolved they are also resistant to biological attack. Specifically tailored/engineered enzymes, however, with advantageous characteristics such as specificity, mild operational conditions (temperature, pressures, acidity and alkalinity, etc.), and potential for in situ application involving no or minimum reactionary hardware can be designed that depolymerize synthetic polymers.

The polymers of choice are liquid crystalline aromatic polyesters and in general contain two or more of the following species; polyethylene-terephthalate (PET), polybutyleneterephthalate (PBT), or Poly-4-Hydroxybenzoic acid and 6-Hydroxy-2-Naphthoic acid. General mechanisms

and models for polyester degradation by hydrolases (lipases) have been devised, evaluated, and published. Previous and ongoing attempts to apply known enzymes to degrade aromatic polyester based TLCPs have not been successful. Readily available lipases/esterases hydrolyze polymers at a rather sharp conformational groove in their structure. The key-lock orientation of the esteratic functionality within the enzymatic catalytic center is the key for catalyzing the depolymerization reaction. However, the flexibility of the polyester chain and, therefore, its capability to accept the hydrolysable polymer sites in the active enzyme center differs dramatically between alkyl and aromatic polyesters. More specifically, as the degree of backbone flexibility within a polymer decreases, the degree of polyester planarity and difficulty to be impacted by the active esterase center increases, accounting for the exceptionally poor biodegradability of highly ordered liquid crystalline polyesters. In this feasibility study the enzymatic depolymerization of the aromatic polyesters (Vectran) was the primary focus of our proposed research.

Recently a new thermophilic hydrolase from *Thermobifida fusca* (TfH) was isolated, characterized and expressed in recombinant *Escherichia coli* [**1, 2**]. The strains of this microorganism are available from the American Type Culture Collection (ATCC). All these microorganisms are BioSafety Level 1 (non-hazardous). This TfH enzyme is especially active in degrading polyesters containing aromatic constituents, and exhibits a 65% sequence similarity to a lipase from *Streptomyces albus* and combines characteristics of lipases and esterases. Currently a range of lipases is available from Novozymes A/S, Denmark, which is one of the largest enzyme manufacturers worldwide. The safety information for the microbial lipases that is provided by Novozymes indicates these enzymes are non toxic.

Most researchers agree that the unique feature of this enzyme's active site is its low degree of indentation (or relatively high planarity), which matches the spatial organization of an aromatic polyester. Available preliminary data on the three dimensional structure of TfH suggest that the cavity of the active site of TfH is not as deep and protected as for other lipases. This structure the need for polymer chain mobility and thus, provides higher degradation rates for high melting aromatic polyesters. Results obtained with the hydrolase from *T. fusca* indicate that aromatic polyesters with melting points above 200°C, such TLCPs, can potentially be depolymerized by an improved hydrolase. Essentially, specific modification of the active site of enzymes like TfH may open the door for enzymatic depolymerization of aromatic polyesters of high crystallinity TLCPs [**2**]. Exploring this feature was the primary goal of this proposed program.

3.2.4.1 Details of Selected Enzyme (Description, Production and Environmental Impact)

TfH enzyme was successfully cloned into *E. coli*, and its recombinant version, rTfH, has been successfully produced, purified, and characterized [**3**]. TfH is classified as a serine hydrolase with the highly conserved G-H-S-M-G motif [**4**]. The enzyme consists of 261 amino acids, corresponding to a molecular weight of 28.2 kDa. Maximum activity was found at 65–70°C. It is active in the purified form and as a biomass crude extract. The rTfH concentrations of 0.5 g L^{-1} can be easily achieved by fermentation. In batch fermentation the major part of the enzyme is accumulated in the extracellular medium, so that its isolation from biomass is facile.

To date, TfH baseline capabilities to degrade aromatic polyesters have been studied using some commercial PET fibers and a model substrate, bis (benzoyloxyethyl) terephthalate (bBOET), Figure 3.

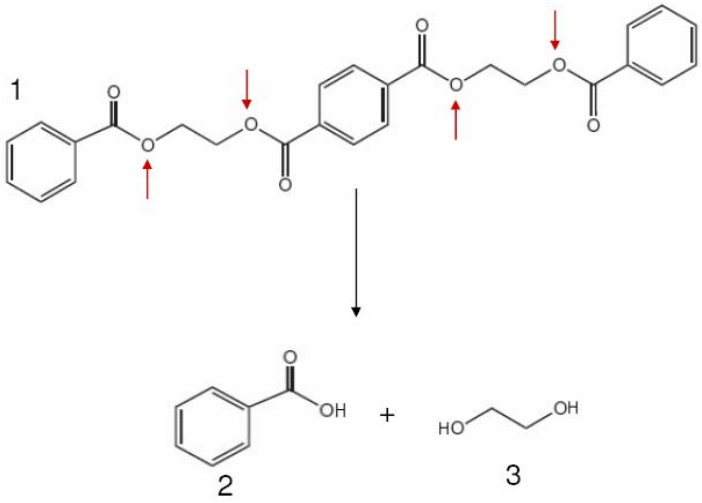

Figure 3 - Model substrate for PET degrading enzymes. Partial substrate degradation (release of glycol and benzoic acid) is shown. Red arrow indicate the hydrolysable esteratic bonds 1 – bBOET; 2- Benzoic acid; 3- Glycol

Figure 3 illustrates the products of polymer degradation, which include benzoic acid and glycol. When a PET based polymer is concerned, the ultimate degradation products would include terephthalate and a glycol (butyleneglycol or ethyleneglycol), Figure 4.

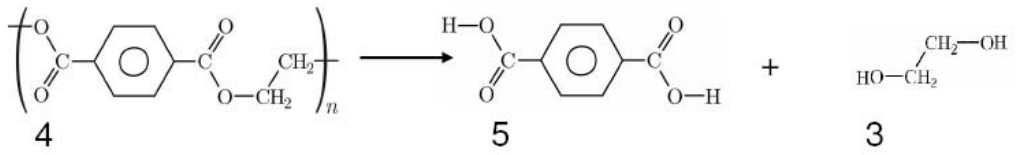

Figure 4 - Schematics of PET hydrolysis 4- PET monomer; 5- Terephthalic acid.

The environmental fate and toxicity of products of biodegradation of a commercially available PET based polymer (Ecoflex, produced by BASF, Germany) treated by the TfH producing strain during composting, including mixtures of terephthalate, terepthalate oligomers, and 1,4-butanediol, has been addressed [**5**]. A toxicological study showed that no significant toxicological effect was observed, for either the monomeric intermediates or for the oligomeric intermediates. During a 30-day composting period the polymer samples were 99.9 % degraded. From a risk assessment it was concluded that there was no indication for an environmental risk when aliphatic-aromatic copolyesters of the Ecoflex-type were introduced into composting processes, suggesting that further disposal of composting products into regular soil and their access to ground waters should be environmentally safe.

In this proposed effort enzyme modification will also focus on decreasing the enzyme's optimum operating temperature to regular ambient conditions.

4. MATERIALS AND METHODS

Thermobifida fusca (TfH) Culture. The microorganism was obtained from the American Type Culture Collection (ATCC). *Thermobifida fusca* 27730 was obtained from the American Type Culture Collection (ATCC, Mannasas, VA). The strain was cultured according to the supplier recommended conditions, such as 50 °C, gentle shaking, and using ATCC medium #741 (ingredients obtained from Sigma-Aldrich) containing tryptone, 3.0 g; yeast extract, 3.0 g; glucose, 3.0 g; K_2HPO_4, 1.0 g; DI water; 1.0 L, pH 7.4, autoclaved 15 minutes at 121 °C. The culture was maintained aseptically in a 0.5 L Erlenmeyer aerobic flask shaken in a Lab-Line shaker-incubator at 120 rpm. Culture volume was 200 mL. The culture period was 7 days.

Mutagen Treatment. The mutagen treatment was performed as follows. The wet biomass (1.35 g) was suspended in 15 mL of phosphate buffered saline pH 8.0 (PBS) in a 50-mL centrifuge tube

capped and centrifuged at 3,000 g. This procedure was repeated twice. To the remaining biomass pellet 15 mL of 1 mg/mL solution of N-ethyl-N-nitrosourea (mutagen) was added. The mixture was vortexed until it became homogeneous. After a 15-min incubation at room temperature the suspension was centrifuged-resuspended-centrifuged as above to remove the mutagen. The mutagen treated biomass was immediately transferred into 200 mL of the ATCC medium #741 for recuperation. All the procedures were performed under aceptic conditions in the BioSafety Class II biocabinet. The treated culture was placed in the Lab-Line shaker-incubator and incubated as above for 7 days.

***Continuous Culture*.** The culture was performed in the continuous flow installation comprised of orbital shaker placed in a 50 °C thermostat, syringe pump to deliver carbon source free mineral medium, silicon tubing lines for fresh medium delivery and used medium transport, and peristaltic pump and plugged flask to collect the used medium. The level of the collection tubing inlet was adjusted inside the culture flask at approximately 200 mL mark, and the peristaltic pump flow rate was set higher than that of the feeding syringe pump; so that the culture volume was maintained constant during the experiment. A specimen of Vectra TLCP film was introduced in the liquid medium to serve as an attachment substrate and carbon source for the microorganism.

Determination of the TLCP degradation activity by culture supernatants

A. TfH free filtrates of the culture liquids were collected by passing the culture liquid supernatants through the Centricon filters (10,000 Da cut-off).
B. TfH containing culture supernatants were collected by centrifuging culture liquids at 25°C and 12,000g for 60 min.

To prevent potential microbial growth the collected liquids were added with sodium azide to the concentration of 0.05 g/mL. These test liquids were incubated aseptically with the TLCP specimens in the Lab-line shaker. The specimens were periodically removed to assess possible changes in their mechanical properties.

Generation of epPCR Mutants. The following microbial strains were used:

- *E. coli* K12 Top10/pCYTEXP1-OmpA-matureBTA1-H_6 - Hydrolase #1, 10/27/08 – Wild Type
- *E. coli* K12 Top10/pCYTEXP1-H_6 - Hydrolase #1, 10/27/08 – Negative Control
- *E. coli* K12 Top10/pCYTEXP1-OmpA-matureBTA1-H_6 epPCR mutants

Random mutations in the hydrolase encoding gene (*bta1*) locus were introduced via epPCR using the GeneMorph® II Random Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All epPCR in this study were performed in a C1000™ thermal cycler (BioRad, Hercules, CA) in 50 µL total volume. The hydrolase *bta1* of *Thermobifida fusca*, hosted in pCYTEXP1-OmpA-matureBTA1-H_6 (courtesy of Dr. Heuvel, Germany), was used as a template along with primers 5'-atctctcacctaccaaacaatgc-3' and 5'-ctattctcactccgctgaaactg-3'. The epPCR conditions were 2 min of pre-denaturation at 95°C, followed by 33 cycles of 30 sec of denaturation at 95°C, 30 sec of annealing at 57°C, and 2 min of extension at 72°C, and then a single final extension of 10 min at 72°C. The epPCR products were double digested with restriction enzymes NdeI and BamH1 and ligated into the respective restriction sites of pCYTEXP1-OmpA-matureBTA1-H_6 (from which WT *bta1* was removed by restriction digestion with NdeI and BamHI). The ligation mixture was electroporated into *E. coli* Top10 (Invitrogen) and transformants were selected LB-Amp500 (500 µg/mL) agar plates. Ampicillin resistant colonies were screened for increased hydrolase activity as described below.

Preliminary Mutant Screening. The epPCR mutants were streaked on LB-Amp500 agar plates and grown overnight at 37°C, then stored at 4°C throughout the screening process. Single epPCR mutant colonies were harvested, spotted onto agar LB-Amp500, and inoculated into 200 µL of LB-Amp500 in a 96-well plate. The solid agar plates, which serves as the mutant clone library, were grown overnight at 37°C and then stored at 4°C. The liquid plates were grown for 5 hr at 39°C and 250 rpm to induce hyrdolase production. Immediately before screening, the plates were mixed vigorously for 50 sec and the cell density for each well obtained with the plate reader (OD_{600}). Screening was performed at room temperature by adding 20 µL of the culture to 180 µL of assay reaction mixture (below), mixing vigorously with the plate reader for 5 sec, and then recording the A_{410} every 30 sec for 10 min. A reference A_{600} was also obtained at each time point. Triplicates of the wild type and negative controls (fresh, grown overnight on LB-Amp500 agar plates) and triplicate blank wells were included in each plate. A pseudo enzymatic activity was used to screen the mutants, and was determined based on the slope of A_{410} vs. time for each well normalized to the wild type with the largest A_{410nm} vs. time slope. Promising mutants were further assayed for enzyme activity as described below.

Enzyme Activity Assay

Purification of Mutants: The epPCR mutants identified for further analysis via screening were purified by streaking from the library plate onto LB-Amp500 agar plates and growing overnight at 37°C.

Culturing: Overnight cultures of the epPCR mutants were prepared by inoculating 25 mL of LB-Amp500 from a single, fresh colony from the purification plate. (Note: Overnight cultures of the five mutants which were analyzed with the Shimadzu 1601 spectrophotometer (Table 2) were inoculated directly from the library plate.) The overnight cultures for the wild type and negative controls were inoculated with a single colony from fresh (grown overnight) LB-Amp500 agar plates streaked from glycerol stocks. Overnight cultures were grown at 37°C and 250 rpm for 16 hr. Assay cultures were prepared by inoculating 250 µL of the overnight culture into 25 mL of LB-Amp500. The assay cultures were grown at 39°C and 250 rpm for 5 hr.

p-Nitrophenyl palmitate Assay: After incubation at 39°C, the assay flasks were transferred to ice and the cell density (OD_{600nm}) was determined, diluting with LB-Amp500 if necessary. A cell pellet was obtained from 1 mL of culture via centrifugation at 13,000 rpm for 1 min. The supernatant (750 µL) were transferred to a sterile 1.5 mL tube and stored on ice until used for assay. Culture supernatant was added to the assay reaction mixture containing the *p*-Nitrophenyl palmitate (pNPP) at a 1:9 ratio (60 µL in 540 µL) in a glass test tube, immediately mixed well, and then assayed in the Shimadzu UV-1601 spectrophotometer by measuring the absorbance at 410 nm (A_{410}) at 30°C at 5 or 30 sec intervals. The A_{410} readings were plotted as a function of time and the slope (A_{410}/min) determined for the linear region of the data. The slope was normalized to the cell density and the mean, standard deviation, and relative error (standard deviation / mean) calculated and used to determine the most appropriate host. Three biological replicates were performed for each mutant. Additionally, two technical replicates of the two randomly chosen samples were performed, one at the beginning and one at the end of the assays, to verify that storage at 4°C for the duration of the assays (approximately 2 hr) was appropriate.

Assay Reaction Mixture

- Phosphate Buffer (0.02M, pH 7.1)
    - Mix 6.7 mL of 0.2M dibasic sodium phosphate solution, 3.3 mL of 0.2M monobasic sodium phosphate solution, and 90 mL double distilled water (100 mL total)

- pNPP Substrate Stock Solution (3.0 g/L)
    - Dissolve 0.03 g pNPP in 10 mL isopropanol
- Reaction Mixture
    - Add 0.23 g of sodium deoxycholic acid and 0.11 g of gum arabic to 100 mL of the phosphate buffer
    - Add 1 mL of stock pNPP solution to 9 mL of Sorensen phosphate buffer with sodium deoxycholic acid and gum Arabic
    - Assay reaction mixture

Sequencing Procedure. Sequencing was performed according to the TKW lab procedure established at the Texas A&M University (TAMU) 'Protocol for DNA sequencing at TAMU'. Primers used for sequencing were forward PCYTEXP1-F2 (5'-gatactgagcacatcagcaggac-3') and reverse PCYTEXP1-R1(5'-ctattctcactccgctgaaactg-3'). Mutations were identified by comparing the mutant sequence to the wild type ompA-mature *bta1*-H_6.

Baseline Hydrolase Activity

Strains

- Wild Type / Positive Control
    - *E. coli* K12 Top10/pCYTEXP1-OmpA-matureBTA1-H_6, Hydrolase #1, 10/27/08
- Negative Control
    - *E. coli* K12 Top10/pCYTEXP1-H_6, Hydrolase #1, 10/27/08

Culture Preparation

Glycerol stocks of both strains were plated on LB-Amp500 agar plates and grown overnight at 37°C. Overnight cultures were prepared by inoculating 25 mL of LB-Amp500 with a single, fresh colony from the agar plate and growing at 37°C and 250 rpm. Cultures used for protein extraction were prepared by inoculating 500 uL of overnight culture into 25 mL of LB-Amp500 and growing to OD600 $\underline{\sim}$ 0.6 at 37°C and 250 rpm (approximately 2 hr), then switching to 39°C and 250 rpm for an additional 5.5 hr.

Crude Protein Extraction

After 5.5 hr of growth at 39°C, the hydrolase was extracted from the culture supernatant by centrifugation (7000 g, 15 min, 4°C) and then 25 uL of protease inhibitor (Sigma Cocktail Mix)

was added to the supernatant. The cell pellets were resuspended in 2 mL of PBS and 20 uL of protease inhibitor, sonicated three times for 30 sec with a 2 min break between each sonication. The sonicated cells were then heat treated at 55°C for 10 min, followed by centrifugation (13,000g, 15 min, 4°C) to remove cell debris. The supernatant was mixed with the initial culture supernatant to form the crude enzyme solution.

Shake Tube Degradation Test

Samples of five different PET materials were evaluated as substrates for hydrolase degradation. A ~25 mg sample of each material was prepared by cutting to size with scissors (if necessary), the dry starting mass obtained, and then transferred to a 10 mL sterile glass test tube. Each sample was then rinsed with sterile de-ionized (DI) water, dried at 80°C for 2 hr, then rinsed with PBS and dried for an additional 1 hr at 80°C. The crude enzyme solution (2 mL) was added to each tube, the tubes were covered with aluminum foil, and then placed at 55°C and ~60 rpm.

After the incubation, the liquid solution was poured off and each sample was rinsed with sterile DI water, rinsed with 95% ethanol, and dried for 6 hr at 80°C.

Determination of TLCP Mechanical Properties

The LCP mechanical testing was conducted using the 5500 Series Instron Mechanical Test Machine at Infoscitex. All tension trials were completed using the guidelines set forth in ASTM D882-Standard Test Method for Tensile Properties of Thin Plastic Sheeting. Sample test area is restricted to 0.5" X 0.5" and each specimen was loaded in tension at a rate of 0.4875"/min. This loading rate of the test material resulted in specimen failure between 30 and 60 seconds. All specimen tests resulted in catastrophic failure of the material being tested. No slipping of the fixture or the specimen was noted. Load was measured using a load cell capable of measuring 0-200N. The temperature and humidity of the test machine and the surrounding environment were held constant for all tests conducted.

5. RESULTS AND ACCOMPLISHMENTS

5.1. Effect of cloned TfH on TLCP

The test polymers were obtained from different sources and included high and low tensile strength PET-based TLCP (Table 2).
The dry mass of each TLCP sample was obtained (Table 2), and then fresh crude enzyme solution (2 mL) was added to each shake tube. An additional 3 mL of PBS solution was added to each shake tube to ensure proper pH was maintained. The samples were again covered with aluminum foil, and then placed at 55°C and ~60 rpm for 8 additional days. This process was repeated once more for a total of 23 days exposure.

For the wild type strain, a total of four replicate shake tubes were prepared (two technical replicates of two independent biological replicates). For the negative control strain, two independent biological replicates were prepared. For the sterile control (PBS only), a single shake tube was prepared.

The activity of the crude enzyme solution was verified visually using the pNPP-based epPCR mutant screening method. In all cases, an immediate color change (enzyme activity) was observed for the crude enzyme solution from the wild type cultures and no color change (no enzyme activity) was observed for the negative control cultures and the PBS solution.

Degradation percentages (Figure 5) were calculated as the difference in mass of the TLCP material divided by the starting mass:

$$\%Degradation = \frac{OriginalMass - FinalMass}{OriginalMass} * 100\% \quad \text{(Equation 1)}$$

Table 2. Description of materials evaluated.

Abbreviation	Chemical Name	Form
Tripp (Tripp Plastics, Sparks, NV)	Melinex 561	Film of middle strength TLCP
Sigma-Aldrich	Poly(4-hydroxybenzoic acid-co-6-hydroxy-2-naphthoic acid)	Flattened pellet of Vectran
Rogers (Rogers, Rogers Corp., Chandler, AZ)	2-Naphthalene carboxylicacid-6-(acetyloxy)-Polymer with 4-(Acetyloxy) Benzoic Acid	Vectran Film
Terphane (Terphane Inc., Bloomfield, New York, NY)	Polyethylene terephthalate homopolymer	Film (Low Strength TLCP)
Coke Coke Zero bottle (vending machine)	Unknown PET-Type TLCP	Film (Low Strength TLCP)

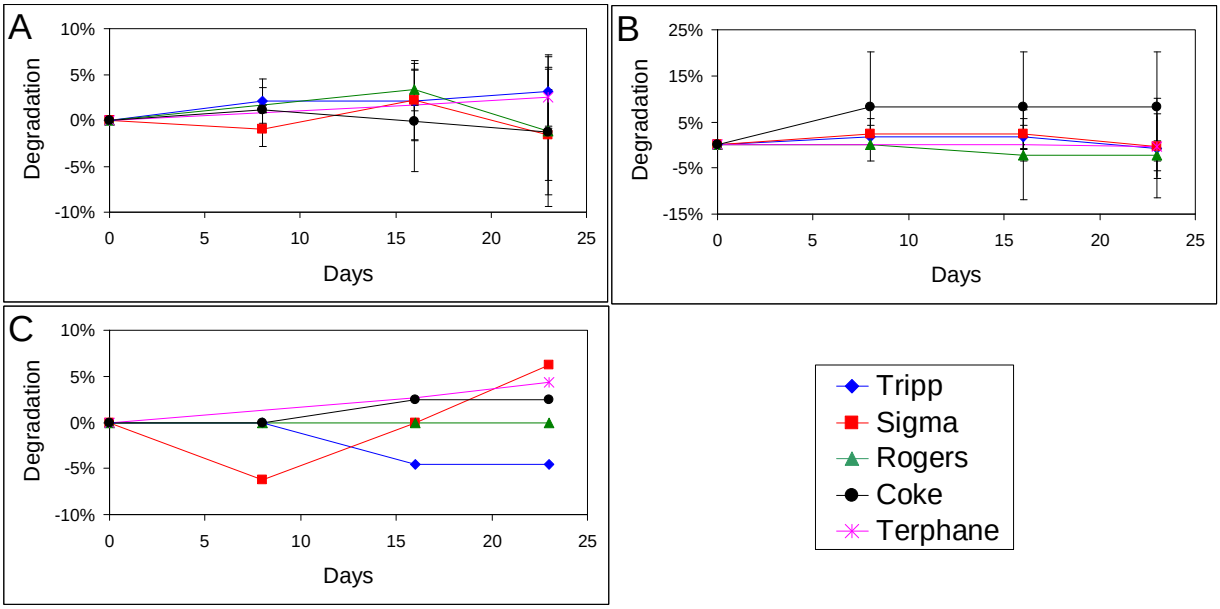

Figure 5. Degradation of the TLCP samples. Each sample was submerged in 2 mL of a crude enzyme solution extracted from liquid cultures of (A) wild type, (B) negative control, or (C) PBS only, and were maintained at 55°C and ~60 rpm. The crude enzyme solution was replaced with fresh solution at the time points corresponding to each data point (8, 16, and 23 days).

Table 3. **Dry mass (g) of each of the TLCP samples (raw data).**

Day 0							
	Positive Control #1		Positive Control #2		Negative Control		
Material	a	b	a	b	#1	#2	PBS Only
Tripp	0.025	0.023	0.024	0.024	0.024	0.029	0.022
Sigma	0.023	0.026	0.028	0.014	0.019	0.021	0.016
Rogers	0.023	0.024	0.021	0.021	0.021	0.022	0.019
Terphane	0.017	0.031	0.030	0.029	0.024	0.020	0.023
Coke	0.021	0.021	0.021	0.020	0.026	0.024	0.040
Day 8							
	Positive Control #1		Positive Control #2		Negative Control		
Material	a	b	a	b	#1	#2	PBS Only
Tripp	0.025	0.023	0.023	0.023	0.024	0.028	0.022
Sigma	0.023	0.027	0.028	0.014	0.019	0.020	0.017
*Rogers	0.023	0.027	0.022	0.036	0.020	0.022	0.019
*Terphane	0.019	0.038	0.032	0.057	0.039	0.023	0.039
Coke	0.021	0.020	0.021	0.020	0.026	0.020	0.040
* Thin film samples were not completely dry, so masses are not accurate							
Day 16							
	Positive Control #1		Positive Control #2		Negative Control		
Material	a	b	a	b	#1	#2	PBS Only
Tripp	0.025	0.023	0.022	0.024	0.024	0.028	0.023
Sigma	0.021	0.026	0.028	0.014	0.019	0.020	0.016
Rogers	0.022	0.023	0.020	0.021	0.020	0.024	0.019
[+]Terphane	0.016	0.028	0.029	0.027	0.023	0.020	0.022
Coke	0.020	0.022	0.020	0.021	0.026	0.020	0.039
[+]A portion of the sample stuck to the shake tube wall during the drying process, thus the masses reported are not accurate.							
Day 23							
	Positive Control #1		Positive Control #2		Negative Control		
Material	a	b	a	b	#1	#2	PBS Only
Tripp	0.025	0.023	0.022	0.023	0.026	0.027	0.023
Sigma	0.022	0.026	0.029	0.015	0.020	0.020	0.015
Rogers	0.022	0.025	0.023	0.020	0.021	0.023	0.019
Terphane	0.017	0.029	0.030	0.028	0.023	0.021	0.022
Coke	0.019	0.023	0.021	0.021	0.026	0.020	0.039

As shown in Figure 5 and Table 3, while some tendency in degradation of lower strength TLCP existed, no pronounced differences in mass loss was observed with any of the compared TLCPs.

5.2 Hydrolase genetic improvement

Comparison of the enzymatic activity before and after two hour storage at 4°C of the wild type and a mutant P35_A12 (randomly selected). Culture supernatant was removed from the cell pellet and stored on ice. The enzyme was clearly stable under these storage conditions based on the very small relative errors for both strains (Table 4).

Table 4. Stability of the enzymatic activity

Sample	Time Stored at 4°C	OD_{600}	ΔA_{410} / min	*Enzymatic Activity (nmol/min·mg)			
				Rep	Mean	Standard Deviation	Relative Error
W/T	0 hr	3.616	0.297	19.8	20.0	0.3	1%
	2 hr	3.616	0.303	20.2			
P35_A12	0 hr	0.820	0.174	51.0	50	2	4%
	2 hr	0.820	0.164	48.2			

The enzymatic activity of the generated clones is presented in Table 5. The clone sequences are presented in Figure 6.

Table 5. Enzymatic activity assay results for selected epPCR mutants. The best three four mutants are highlighted in green. Mutant P7_F03, highlighted in green, is the fourth best mutant and has 2.9-fold increase in activity relative to the wild type strain; mutant P7_F03 has been sequenced (Figure 6). Because multiple spectrophotometers were used, the fold change in enzymatic activity is relative to the wild type assayed on the same day using the same instrument. (Note: All further analysis was performed using the Shimadzu 1601 spectrophotometer.)

Spectrophotometer	Sample	Bio Rep	OD_{600}	ΔA_{410} / min	*Enzymatic Activity (nmol/min·mg)				
					Rep	Mean	Standard Deviation	Relative Error	Fold Change
Cary E1	W/T	1	0.607	0.214	84.7	89	15	17%	1.0
		2	0.594	0.188	76.2				
		3	0.640	0.280	105.4				
	P1_A1	1	0.585	0.171	70.1	109	34	31%	1.2
		2	0.682	0.368	129.5				
		3	0.678	0.358	126.9				
	P3_C9	1	0.603	0.206	82.0	86	4	4%	1.0
		2	0.613	0.227	89.0				
		3	0.611	0.222	87.4				
	P6_B8	1	0.541	0.082	36.6	26	10	38%	0.3
		2	0.528	0.057	26.0				
		3	0.517	0.035	16.5				
	Neg Cont	1	0.282	0.001	0.8				

Shimadzu BioMini	W/T	1	0.806	0.070	20.9	24	10	40%	1.0
		2	0.584	0.084	34.7				
		3	1.060	0.072	16.3				
	P1_F7	1	0.508	0.088	41.5	26	14	54%	1.1
		2	1.006	0.078	18.7				
		3	1.418	0.097	16.4				
	P7_F9	1	0.570	0.059	25.0	26	3	10%	1.1
		2	0.508	0.060	28.3				
		3	0.594	0.058	23.3				
	P7_F3	1	0.316	0.061	46.6	69	28	40%	2.9
		2	0.388	0.162	100.4				
		3	0.540	0.137	61.1				
	Neg Cont	1	3.605	0.003	0.2				
Shimadzu 1601	W/T	1	2.676	0.328	29.5	22	6	29%	1.0
		2	3.616	0.297	19.8				
		3	3.672	0.263	17.2				
	Neg Cont	1	3.768	0.000	0.0	0	0	0%	--
		2	3.848	0.000	0.0				
		3	3.832	0.000	0.0				
	P39_G08	1	1.184	0.196	39.8	40	9	21%	1.8
		2	2.032	0.266	31.5				
		3	0.856	0.173	48.6				
	P35_A12	1	0.596	0.198	79.9	62	16	25%	2.8
		2	0.820	0.173	50.7				
		3	0.688	0.160	56.0				
	P25_D03	1	0.516	0.197	91.7	102	9	9%	4.6
		2	0.484	0.212	105.0				
		3	0.436	0.197	108.5				
	P38_F03	1	0.408	0.188	110.9	75	36	48%	3.4
		2	0.524	0.164	75.2				
		3	1.248	0.199	38.4				
	P40_B11	1	0.476	0.168	85.0	78	10	13%	3.5
		2	0.568	0.156	66.1				
		3	0.496	0.169	81.7				

* Based on ε = 10,400 L/mol·cm (Wilhelm *et al*, 1999) and 0.24 mg protein/mL·OD unit

DNA Sequence

P7_F3-epbta1

ATGAAAAAGACAGCTATTGCGATTGCAGTGGCACTGGCTGGTTTCGCGACCGTAGCG
CACGCTATGGCCAACCCCTACGAGCGCGGCCCC

WT-bta1

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCGACCGTAGCG
CACGCTATGGCCAACCCCTACGAGCGCGGCCCC

91 180

P7_F3-epbta1
AACCCGACCGACGCCCTGCTCGAAGCCAGCAGCGGCCCCTTCTCCGTCAGCGAGGAG
AACGTCTCCCGGTTGAGCGCCAGCGGCTTCGGC
WT-bta1
AACCCGACCGACGCCCTGCTCGAAGCCAGCAGCGGCCCCTTCTCCGTCAGCGAGGAG
AACGTCTCCCGGTTGAGCGCCAGCGGCTTCGGC
181 270
P7_F3-epbta1
GGCGGCACCATCTACTACCCGCGGGAGAACAACACCTACGGTGCGGTGGCGATCTCC
CCCGGCTACACCGGCACTGAGGCTTCCATCGCC
WT-bta1
GGCGGCACCATCTACTACCCGCGGGAGAACAACACCTACGGTGCGGTGGCGATCTCC
CCCGGCTACACCGGCACTGAGGCTTCCATCGCC
271 360
P7_F3-epbta1
TGGCTGGGCGAGCGCATCGCCTCCCACGGCTTCGTCGTCATCACCATCGACACCATC
ACCACCCTCGACCAGCCGGACAGCCGGGCAGAG
WT-bta1
TGGCTGGGCGAGCGCATCGCCTCCCACGGCTTCGTCGTCATCACCATCGACACCATC
ACCACCCTCGACCAGCCGGACAGCCGGGCAGAG
361 450
P7_F3-epbta1
CAGCTCAACGCCGCGCTGAACCACATGATCAACCGGGCGTCCTCCACGGTGCGCAGC
CGGATCGACAGCAGCCGACTGGCGGTCATGGGC
WT-bta1
CAGCTCAACGCCGCGCTGAACCACATGATCAACCGGGCGTCCTCCACGGTGCGCAGC
CGGATCGACAGCAGCCGACTGGCGGTCATGGGC
451 540
P7_F3-epbta1
CACTCCATGGGCGGCGGCGGCACCCTGCGTCTGGCCTCCCAGCGTCCCGACCTGAAG
GCCGCCATCCCGCTCACCCCGTGGCACCTCAAC
WT-bta1
CACTCCATGGGCGGCGGCGGCACCCTGCGTCTGGCCTCCCAGCGTCCCGACCTGAAG
GCCGCCATCCCGCTCACCCCGTGGCACCTCAAC
541 630
P7_F3-epbta1
AAGAACTGGAGCAGCGTCACCGTGCCGACGCTGATCATCGGGGCCGACCTCGACAC
AATCGCGCCGGTCGCCACGCACGCGAAACCGTTC
WT-bta1
AAGAACTGGAGCAGCGTCACCGTGCCGACGCTGATCATCGGGGCCGACCTCGACAC
AATCGCGCCGGTCGCCACGCACGCGAAACCGTTC
631 720
P7_F3-epbta1
TACAACAGCCTGCCGAGCTCCATCAGCAAGGCCTACCTGGAGCTGGACGGCGCAACC
CACTTCGCCCCGAACATCCCCAACAAGATCATC
WT-bta1
TACAACAGCCTGCCGAGCTCCATCAGCAAGGCCTACCTGGAGCTGGACGGCGCAACC
CACTTCGCCCCGAACATCCCCAACAAGATCATC
721 810

P7_F3-epbta1
GGCAAGTACAGCGTCGCCTGGCTCAAGCGGTTCGTCGACAACGACACCCGCTACACC
CAGTTCCTCTGCCCCGGACCGCGCGACGGACTC
WT-bta1
GGCAAGTACAGCGTCGCCTGGCTCAAGCGGTTCGTCGACAACGACACCCGCTACACC
CAGTTCCTCTGCCCCGGACCGCGCGACGGACTC
811 879
P7_F3-epbta1
TTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCGTTCCTCGAGCACCGCCACCAC
CACCACTGATAA
WT-bta1
TTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCGTTCCTCGAGCACCACCACCAC
CACCACTGATAA

Amino Acid Sequence

Wild Type: ATC=Ile P7-F3 Mutant: ATT=Ile
Wild Type: CAC=His P7-F3 Mutant: CGC=Arg

P7_F3-epbta1
MKKTAIAIAVALAGFATVAQAMANPYERGPNPTDALLEASSGPFSVSEENVSRLSASGFG
GGTIYYPRENNTYGAVAISPGYTGTEASIA
WT-bta1
MKKTAIAIAVALAGFATVAQAMANPYERGPNPTDALLEASSGPFSVSEENVSRLSASGFG
GGTIYYPRENNTYGAVAISPGYTGTEASIA
91 180
P7_F3-epbta1
WLGERIASHGFVVITIDTITTLDQPDSRAEQLNAALNHMINRASSTVRSRIDSSRLAVMGH
SMGGGGTLRLASQRPDLKAAIPLTPWHLN
WT-bta1
WLGERIASHGFVVITIDTITTLDQPDSRAEQLNAALNHMINRASSTVRSRIDSSRLAVMGH
SMGGGGTLRLASQRPDLKAAIPLTPWHLN
181 270
P7_F3-epbta1
KNWSSVTVPTLIIGADLDTIAPVATHAKPFYNSLPSSISKAYLELDGATHFAPNIPNKIIGK
YSVAWLKRFVDNDTRYTQFLCPGPRDGL
WT-bta1
KNWSSVTVPTLIIGADLDTIAPVATHAKPFYNSLPSSISKAYLELDGATHFAPNIPNKIIGK
YSVAWLKRFVDNDTRYTQFLCPGPRDGL
271 293
P7_F3-epbta1 FGEVEEYRSTCPFLEHRHHHH--
WT-bta1 FGEVEEYRSTCPFLEHHHHHH--

Figure 6. DNA and amino acid sequence comparisons of the wild type and the fourth best mutant, P7_F03. The two mutations are highlighted in red, and were observed in the OmpA and His-tag regions rather than in the hydrolase sequence. The OmpA (N-terminus) is highlighted green, and the His-tag is highlighted blue.

Thus, we were able to increase the activity of hydrolase in selected clones up to 4.6-fold as compared to the wild strain. The sequence of these prominent hydrolyses was improved as well.

5.3 Improvement of intracellular enzyme secretion

Improvement of intracellular enzyme secretion was attempted by deletion of the *lpp* gene. The deletion of the *lpp* gene (Braun's lipoprotein) has been shown to increase the secretion rate of the extracellular enzymes [**6**]. In this Phase I project, deletion of the *lpp* gene was applied to improve release of the recombinant hydrolase (rTfH) from the cytoplasm.

Notes:

- *recA* gene was deleted from BW25113 to prevent probable recombination of pCYTEXP1 plasmid into the host genome
- *recA* and *lpp* gene deletions were confirmed by PCR

The strains subjected to the deletion of the *lpp* gene are listed in Table 6.

Table 6. Strains for the *lpp* gene deletion

Strain name	Genotype	Enzyme
US 3011	*E. coli* BW25113 *ΔrecA Δkan* pCYTEXP1-bta1-ompA-H_6	Hydrolase # 1
US 3013	*E. coli* BW25113 *Δlpp ΔrecA Δkan* pCYTEXP1-bta1-ompA-H_6	Hydrolase # 1
US 3014	*E. coli* BW25113 *Δlpp ΔrecA Δkan* pCYTEXP1 (negative control plasmid)	Hydrolase # 1

The activity of the released enzyme upon the deletion of the *lpp* gene is presented in Table 7 and Table 8.

Table 7. Activity of hydrolase enzyme in the pNPP assay at 3 hrs of culturing at 39 °C.

Strain	Rep	OD600	ΔA_{410}/min	Enzyme Activity (nmol/min*mg)				Fold Change
				Enzyme Activity	Average	St Dev	Rel Error	
US 3011	1	1.696	0.023152	3.281501	2.45	0.73	0.30	1.0
	2	1.836	0.016802	2.199871				
	3	1.98	0.015484	1.879877				
US 3013	1	1.632	0.060316	8.884164	5.78	2.76	0.48	2.4
	2	1.912	0.038767	4.873922				
	3	2.032	0.030391	3.595215				
US 3014	--	3.044	-0.00292	-0.23073	--	--	--	--

Table 8. Activity of hydrolase enzyme in the pNPP assay at 5 hrs of culturing at 39°C.

Strain	Rep	OD600	ΔA410/min	Enzyme Activity (nmol/min*mg)				Fold Change
				Enzyme Activity	Ave	StDev	Rel Error	
US 3011	1	4.63	0.660701	34.30287	25.86	7.33	0.28	1.0
	2	4.84	0.445083	22.10558				
	3	5.05	0.444831	21.17435				
US 3013	1	2.44	0.990327	97.56534	76.04	20.55	0.27	2.9
	2	2.89	0.680744	56.62298				
	3	3.11	0.956497	73.93153				
US 3014	1	6.18	0.004897	0.19046	--	--	--	--
	2	6.12	0.000806	0.031643	--	--	--	--

The SDS-PAGEs of the culture supernatants were also run to confirm elevated excretion of the rTfH upon the *lpp* deletion (Figure 7).

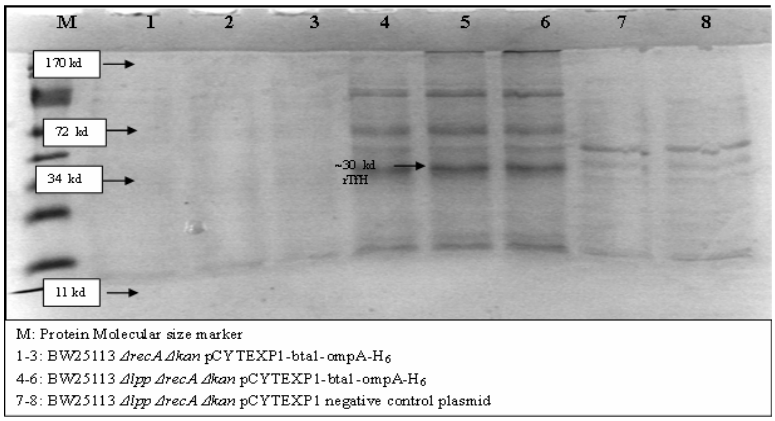

Figure 7. SDS PAGE of the rTfH.

The obtained results indicated that BW25113 *Δlpp ΔrecA Δkan* strain was more efficient than BW25113 *ΔrecA Δkan* strain in secreting of the extracellular protein.

In summary, based on the pNPP assay, deletion of *lpp* gene increased the secretion of hydrolase enzyme by 2.9 times after 5 hrs incubation at 39 °C. Moreover, SDS-PAGE confirmed results obtained with the pNPP assay that supernatant of *E. coli* BW25113 *Δlpp ΔrecA Δkan* strain has higher secreted protein than BW25113 *ΔrecA Δkan*.

5.4 Effect of natural TfH on LCP mechanical properties.

We investigated influence of exposure of the TLCP material (Rodgers Vectra) to natural enzyme solution (culture supernatants) of *Thermobifida fusca* at 50 °C and at room temperature.

It was observed that after a six-month exposure to the enzyme solution at 50 °C some deterioration of the TLCP film mechanical properties did occur. More specifically, the modulus and elongation at break were reduced by 30 % compared to TLCP film immersed into culture supernatant with no enzyme (protein removed by Centricon filtration). Smaller additional decrease in modulus and elongation at break was observed during the TLCP film treatment immersion in the continuous culture of the mutated *Thermobifida fusca*. However, there was no significant change in the TLCP mechanical properties at room temperature (Figure 8). These observations are in agreement with the optimal temperature of *Thermobifida fusca* (65-70 °C).

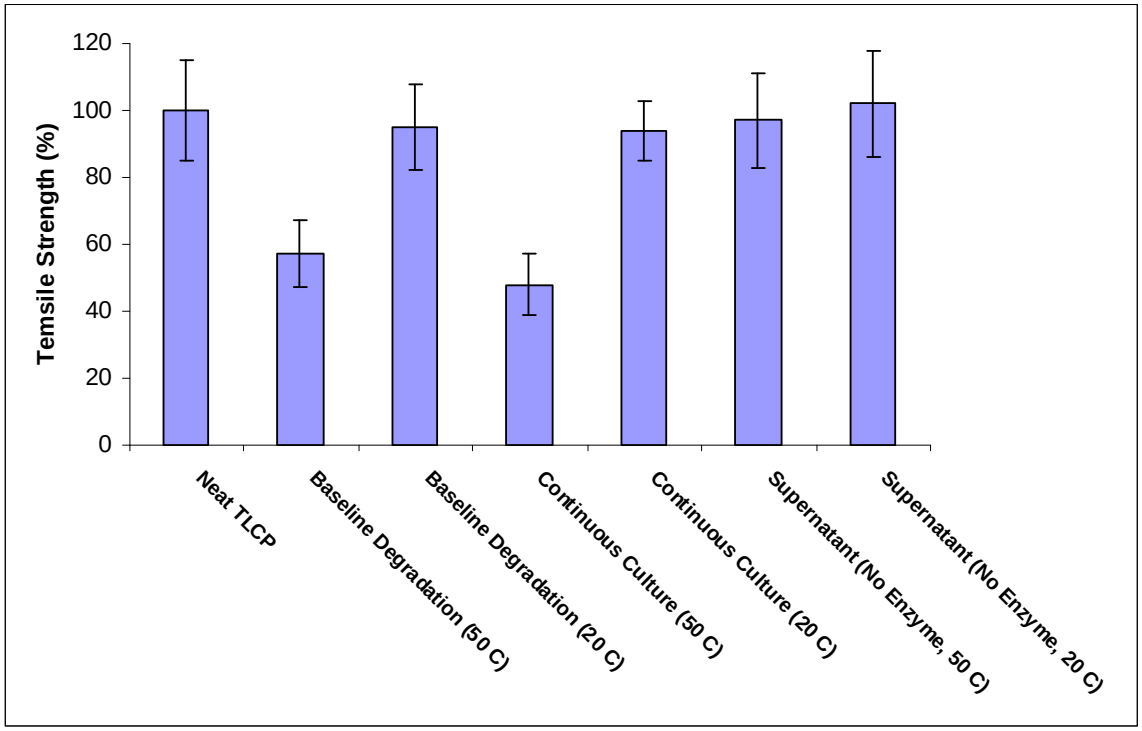

Figure 8. Tensile strength of TLCP as function of exposure to *Thermobifida fusca*

6. CONCLUSIONS and RECOMMENDATIONS

In this Phase I SERDP feasibility study, Infoscitex Corporation and the University of Texas A&M evaluated possibility of improvement of microbial hydrolyses for biodegradation of PET-based TLCP. Based on the experimental results discussed in this report, the following conclusions were made:

- Several *E. coli* clones bearing the original and mutated genes of TfH deriving from Thermobifida *fusca* and exhibiting an improved total hydrolase production and excretion can be successfully generated using error-prone PCR to create beneficial mutations. This system could potentially degrade various polymers, including PET-based TLCP. These strains would provide additional environmental benefits through their application for environmental clean up. The resultant enzymes were relatively stable in solution.
- The potential of enzymatic biodegradation of the TLCP films was evaluated. The enzymatic exposure at 50 °C resulted in significant deterioration of the mechanical properties of the TLCP film. This result needs to be replicated at ambient temperatures to achieve optimal degradation in the field.
- The elevated levels of the hydrolase release from cells were successfully achieved by the *lpp* gene deletion.
- The genetically improved hydrolases exhibited up to 4.6-fold increase in specific enzymatic activity. The increased activity of the improved hydrolases is anticipated to dramatically increase the applied degradation to the TLCP at ambient temperatures.

The conclusions of this study clearly indicate that rapid degradation of a structural polymer is possible, thus making the RDPS a valuable tool to reduce environmental impact from detonated ordnance casings. Future work needs to be done to further develop RDPS and eventually test in live fire validation studies. In the SERDP Phase II project, IST will focus on increasing ambient temperature activity of the hydrolases, designing the enzyme release process and transitioning the technology with ordnance designers and suppliers.

7. REFERENCES

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